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<b>(54) Title:</b> PURIFICATION OF HUMAN INTERLEUKIN-10		
<b>(57) Abstract</b> <p>Disclosed is a method for purifying Interleukin-10 (IL-10). The method is comprised of subjecting an IL-10 containing solution to cation exchange chromatography, anion exchange chromatography, hydroxyapatite chromatography, and gel filtration chromatography. The present invention is also comprised of a process for separating different IL-10 dimers present in a protein fraction from each other by subjecting the protein fraction to hydroxyapatite chromatography. The present invention is also comprised of a process for separating variants of a protein differing in an N-terminal amino acid sequence present in a protein fraction from each other by subjecting the protein fraction to hydroxyapatite chromatography.</p>		

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PURIFICATION OF HUMAN INTERLEUKIN-10Background of the Invention

Interleukin-10 (IL-10), a recently discovered lymphokine, was  
10 originally described as an inhibitor of interferon- $\gamma$  synthesis and is  
postulated as a major mediator of the humoral class of immune  
response [Fiorentino, D.F., *et al.*, *J. Exp. Med.* 170: 2081 (1989) and Moore  
*et al.*, K.W., *et al.*, *Science* 248: 1230-1234 (1990)]. Two classes of often  
mutually exclusive immune responses are the humoral (antibody-  
15 mediated) and the delayed-type hypersensitivity.

It is postulated that these two differing immune responses may  
arise from two types of helper T-cell clones, namely Th1 and Th2 helper  
T-cells, which demonstrate distinct cytokine secretion patterns [Moore  
20 *supra*; Vieira, P. *et al.*, *Proc. Nat. Acad. Sci. USA* Vol. 88: 1172 (1991)].  
Mouse Th1 cell clones secrete interferon- $\gamma$  and IL-2 and preferentially  
induce the delayed-type hypersensitivity response while Th-2 cell clones  
secrete IL-4, IL-5 and IL-10 and provide support for the humoral  
responses [Fiorentino *et al.*, *supra*]. The contrast in immune response  
25 could result because interferon- $\gamma$  secreted by the Th1 cell clones inhibits  
Th2 clone proliferation *in vitro*, while IL-10 secreted by the Th2 cell  
clones inhibit cytokine secretion by the Th1 cell clones [Fiorentino *et al.*,  
*supra* and Moore *et al.*, *supra*]. Thus the two T-helper cell types may be  
mutually inhibitory and may provide the underpinning for the two  
30 dissimilar immune responses.

IL-10 has been cloned and sequenced from both murine and  
human T cells [Moore *et al.*, *supra* ; Vieira *et al.*, *supra*]. Both sequences  
contain an open reading frame encoding a polypeptide of 178 amino  
35 acids with an N-terminal hydrophobic leader sequence of 18 amino  
acids, and have an amino acid sequence homology of 73%.

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Biologically active IL-10 is a dimer as determined by analytical gel filtration. Generally, the dimer is non-covalently bonded based upon its migration as a monomer on non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Recombinant human IL-10 can be expressed both by prokaryotic and eukaryotic expression systems.

N-terminal analysis of recombinant human IL-10 produced in a eukaryotic expression system indicates that a small percentage of IL-10 polypeptides have the first two N-terminal amino acid residues missing. This truncated polypeptide is referred to as the  $\Delta 2$  IL-10 polypeptide, or simply  $\Delta 2$ . The full-length chain is therefore referred to as  $\Delta 0$ , indicating that no amino acid has been deleted. Accordingly, biologically active, eukaryotically expressed IL-10 can appear as three different dimers. The first biologically active dimer and the major form is  $\Delta 0:\Delta 0$ , a homodimer in which both polypeptides of the dimer have the full-length chain of amino acids. The second IL-10 dimer is  $\Delta 0:\Delta 2$ , a heterodimer in which one of the polypeptide chains has the full-length chain of amino acids and the second chain,  $\Delta 2$ , has the first two N-terminal amino acids missing. The third IL-10 dimer is  $\Delta 2:\Delta 2$ , a homodimer in which both polypeptide chains of the dimer have the initial two N-terminal amino acid residues missing. Thus, there is a need for a process to purify IL-10, and in particular there is a need for a process which separates the different dimers of IL-10 from each other.

IL-10 contained in inclusion bodies expressed by a prokaryotic expression system must be, denatured, refolded, and purified from contaminants including host proteins, modified variants of IL-10 and heterodimers of those variants. Furthermore, in a prokaryotic system, the IL-10 monomer can be acetylated at one or more of the lysine residues. If an acetylated monomer binds to another acetylated monomer then an acetylated homodimer is produced. If however, a non-acetylated monomer binds to another non-acetylated monomer then a non-acetylated homodimer is produced. If an acetylated monomer binds to a non-acetylated monomer then a heterodimer is produced. Furthermore, IL-10 is normally produced as a non-covalently bonded homodimer. However, during denaturing of the inclusion bodies, and refolding of the IL-10 a covalently bonded homodimer can be produced, i.e. one which migrates as a dimer on

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non-reducing SDS-PAGE but as a monomer under reducing conditions. This is probably caused by one or more intermolecular disulfide bonds which are formed between the two monomers. Thus, there is a need to purify IL-10 from host protein contaminants and to obtain essentially  
5 pure non-covalently bonded dimeric IL-10 free of the acetylated homodimer, heterodimer variants and covalent dimers.

In light of its role as a potential immune response mediator and its activity as an inhibitor of interferon- $\gamma$  synthesis, IL-10 may have  
10 clinical utility in autoimmune diseases or transplant rejection. However, in a clinical setting it is highly desirable that the IL-10 be in a highly pure state, substantially free of other contaminating host and medium proteins or polypeptides. Thus, there is a need for a process to purify IL-10 which accomplishes these objectives.

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### Summary of the Invention

20 The present invention fills this need by providing a process for purifying IL-10 contained within a solution comprising:

(a) subjecting the solution containing IL-10 to cation exchange chromatography thereby obtaining fractions containing IL-10;

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(b) subjecting the IL-10-containing fractions from step (a) to anion exchange chromatography thereby obtaining fractions containing IL-10;

(c) subjecting the IL-10-containing fractions from step (b) to  
30 hydroxyapatite chromatography thereby obtaining fractions containing a single isolated dimer of IL-10; and

(d) subjecting the IL-10-containing fractions from step (c) to gel-filtration chromatography thereby obtaining IL-10 containing fractions  
35 free of high and low molecular weight impurities.

The purification process of IL-10 can be used for IL-10 expressed in bacterial or eukaryotic expression systems.

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5 The present invention further provides a method for separating different IL-10 dimers contained within a protein fraction containing a mixture of dimers comprising subjecting the fraction to hydroxyapatite chromatography under conditions in which the dimers separate from each other.

10 The present invention further provides a method for separating different dimers of a protein contained within a protein fraction wherein the different dimers have different N-terminal amino acid sequences comprising subjecting the protein fraction to hydroxyapatite chromatography under conditions wherein the different dimers of the protein are separated from each other.

15 The present invention further provides a method for separating variants of a protein contained within a protein fraction wherein the variants of the protein have different N-terminal amino acid sequences comprising subjecting the protein fraction to hydroxyapatite chromatography under conditions wherein the variants of the protein  
20 are separated from each other.

The present invention still further provides a method for separating non-acetylated homodimers of IL-10 from acetylated homodimers and from acetylated heterodimers of IL-10 contained  
25 within a solution comprising subjecting the solution to anion exchange chromatography under conditions in which the non-acetylated homodimer is separated from the acetylated dimers of IL-10.

### Description of the Invention

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All references cited herein are hereby incorporated in their entirety by reference.

35 As used herein, "interleukin-10" or "IL-10" can be either human IL-10 (h IL-10) or murine IL-10. Human IL-10 is defined as a protein which (a) has an amino acid sequence substantially identical to a known sequence of mature (i.e., lacking a secretory leader sequence) hIL-10 as disclosed in U.S. Patent Application Serial No. 07/917,806, filed July 20,

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1992, which corresponds to International Application No. PCT/US90/03554, Publication No. WO 91/00349, and (b) has biological activity that is common to native hIL-10.

5 IL-10 can be obtained from culture media of activated T-cells capable of secreting the protein. Preferentially, however, it is obtained by recombinant techniques using isolated nucleic acids encoding for the IL-10 polypeptide. General methods of molecular biology are described, e.g., by Sambrook, *et al.*, *Molecular Cloning, A Laboratory Manual*, Cold  
10 Spring Harbor Publish., Cold Spring Harbor, New York, 2d ed. 1989 and by Ausubel *et al.*, (eds.) *Current Protocols in Molecular Biology*, Green/Wiley, New York (1987 and periodic supplements). The appropriate sequences can be obtained from either genomic or cDNA libraries. Polymerase chain reaction (PCR) techniques can be used. See,  
15 e.g., *PCR Protocols: A Guide to Methods and Applications*, 1990, Innis *et al.*, (Ed.), Academic Press, New York, New York.

Libraries are constructed from nucleic acid extracted from appropriate cells. See, for example, International Application  
20 Publication No. WO 91/00349, which discloses recombinant methods to make IL-10. Useful gene sequences can be found, e.g., in various sequence data bases, e.g., Gen Bank and EMBL for nucleic acid, and PIR and Swiss-Prot for protein, c/o Intelligenetics, Mountain View, California, or the Genetics Computer Group, University of Wisconsin  
25 Biotechnology Center, Madison, Wisconsin, which are incorporated herein by reference.

Clones comprising sequences that encode human IL-10 (hIL-10) have been deposited with the American Type Culture Collection  
30 (ATCC), Rockville, Maryland, under Accession Numbers 68191 and 68192. Identification of other clones harboring the sequences encoding IL-10 is performed by either nucleic acid hybridization or immunological detection of the encoded protein, if an expression vector is used. Oligonucleotide probes based on the deposited sequences are  
35 disclosed in International Application Publication No. WO 91/00349. Oligonucleotide probes useful for identification of the sequences can also be prepared from conserved regions of related genes in other

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species. Alternatively, degenerate probes based on the amino acid sequence of IL-10 can be used.

Various expression vectors can be used to express DNA encoding IL-10. Conventional vectors used for expression of recombinant proteins used for expression of recombinant proteins in prokaryotic or eukaryotic cells may be used. Preferred vectors include the pcD vectors described by Okayama *et al.*, Mol. Cell. Bio. Vol. 3: 280-289 (1983); and Takebe *et al.*, Mol. Cell. Biol. Vol. 8: 466-472 (1988). Other SV40-based mammalian expression vectors include those disclosed in Kaufman *et al.*, Mol. Cell. Biol. Vol. 2: 1304-1319 (1982) and U.S. Patent No. 4,675,285. These SV40-based vectors are particularly useful in COS7 monkey cells (ATCC No. CRL 1651), as well as in other mammalian cells such as mouse L cells and CHO cells.

Standard transfection methods can be used to produce eukaryotic cell lines which express large quantities of the polypeptide. The process of the present invention is a process to purify IL-10 expressed by eukaryotic cells from a cell supernatant into which the protein was expressed. Eukaryotic cell lines include mammalian, yeast and insect cell lines. Exemplary mammalian cell lines include COS-7 cells, mouse L cells and Chinese Hamster Ovary (CHO) cells. See Sambrook *et al.*, *supra* and Ausubel *et al.*, *supra*.

Furthermore, the method of the present invention provides for a method to purify IL-10 produced by genetically transformed bacteria, particularly *E. coli*. As used herein, the term "transformed bacteria" means bacteria that have been genetically engineered to produce a mammalian protein. Such genetic engineering usually entails the introduction of an expression vector into a bacterium. The expression vector is capable of autonomous replication and protein expression relative to genes in the bacterial genome. Construction of bacterial expression is well known in the art, provided the nucleotide sequence encoding a desired protein is known or otherwise available. For example, DeBoer in U.S. Pat. No. 4,551,433 discloses promoters for use in bacterial expression vectors; Goeddel *et al.* in U.S. Pat. No. 4,601,980 and Riggs, in U.S. Pat. No. 4,431,739 disclose the production of mammalian proteins by *E. coli* expression systems;



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and Riggs *supra*, Ferretti *et al. Proc. Natl. Acad. Sci.*83:599 (1986), Sproat *et al., Nucleic Acid Research* 13:2959 (1985) and Mullenbach *et al., J. Biol. Chem* 261:719 (1986) disclose how to construct synthetic genes for expression in bacteria. Many bacterial expression vectors are available  
5 commercially and through the American Type Culture Collection (ATCC), Rockville, Maryland.

The method of the present invention comprises the sequential application of cation-exchange, anion-exchange, hydroxyapatite and gel-  
10 filtration chromatography. To achieve high purity and maximal yield, each of the four chromatographic steps were selected and optimized in regards to pH, conductivity, buffer composition, flow rates and column dimensions. The analytic procedures utilized to determine this optimization of purity and yield were Western blots, sodium dodecyl  
15 sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) Laemmli, U.K., *Nature* 227:680 (1970), Enzyme-Linked Immunoabsorbant Assay (ELISA) values, UV absorbance at 280 and 260 nm and protein concentration determinations [(Bradford, M., *Anal. Biochem.*,72: 248 (1976)].

20 Furthermore, the order of chromatographic steps were optimized for efficient and rapid large-scale processing as well as product purity and yield. This includes 1) product concentration during the first step (cation exchange) to reduce volume handling; 2) a flow-through mode for the second step (anion exchange) such that the product of this fast  
25 step can immediately be loaded on the next column without extra processing; 3) the removal of almost all contaminating proteins during the first two steps so that there is no interference by these other proteins with the resolution of the IL-10 forms on the third column (hydroxyapatite) and 4) besides separation of trace contaminants of  
30 differing molecular weights and IL-10 monomer, the buffer exchange of the gel filtration chromatography allows the final product IL-10 to be obtained in a buffer desired for further pharmaceutical formulation.

The cation exchange chromatography step is used first because  
35 IL-10 adsorbs well to a cation exchange resin. Any cationic exchange group can be used such as carboxymethyl, sulfopropyl and sulfonate. The cationic exchange group can be attached to any solid-phase support including but not limited to cellulose, dextrans, agarose and polystyrene.

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The preferred cation exchange group is sulfonate attached to an agarose packing support matrix, such as S-SEPHAROSE Fast Flow® from (Pharmacia, Piscataway, N.J.). The equilibrating buffer should be at a pH lower than 7.8, the pI of IL-10, and preferably about 6.5 for S-  
5 SEPHAROSE, in order to create sufficient positive charges on the IL-10 protein. This creates good adhesion of the IL-10 protein to the cationic exchange group.

If the IL-10 is produced by a mammalian cell culture expression  
10 system, conditions are optimized such that 80 - 90% of contaminant proteins, and especially serum albumin, the major contaminant protein, are not bound. The amount of protein that is to be loaded can be determined experimentally based upon information provided by the manufacturer. Using a 5 x 28 cm S-SEPHAROSE® Fast  
15 Flow column, approximately 100 mg protein/ml of bed volume can be applied at a flow rate of 1.1 cm/min. After the supernatant is loaded, the column is developed with either a step or linear gradient salt solution, preferably a 70-300 mM NaCl linear gradient for an S-SEPHAROSE® column. IL-10 elutes at a concentration of about 150 mM  
20 NaCl at a distinct peak of A<sub>280</sub> at approximately 17 mS. Ideally IL-10 containing fractions are then concentrated and diafiltered by, for example, using a PELLICON®, 10K membrane.

If IL-10 is produced in inclusion bodies in a bacterial  
25 expression system, the IL-10 is generally denatured and then refolded. A solution containing the refolded IL-10 is then applied to the cation exchange resin as described above. Conditions are optimized such that 80 - 90% of contaminant proteins are not bound. The amount of protein that is to be loaded can be determined experimentally based upon  
30 information provided by the manufacturer. Using a 12 x 36 cm S-SEPHAROSE® Fast Flow column, approximately 15 mg protein/ml of bed volume can be applied at a flow rate of 1 cm/min. After the supernatant is loaded, the column is developed with either a step or linear gradient salt solution, preferably a 65-400 mM NaCl linear  
35 gradient for an S-SEPHAROSE® column. IL-10 elutes at a concentration of about 150 mM NaCl at a distinct peak of A<sub>280</sub> at approximately 17 mS. Ideally IL-10 containing fractions are then concentrated and diafiltered by, for example, using a PELLICON®, 10K membrane.

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The IL-10-containing fractions from the cation exchange chromatography are subjected to anion exchange chromatography which substantially removes the remaining host or cell culture protein contaminants. Any anionic exchange group can be used. Examples are quaternary aminoethyl, mixed amine or other intermediate base or weak base exchange groups. Quaternary aminoethyl is a preferred anion exchange group. The quaternary aminoethyl group may be attached to a dextran, cellulose, agarose or acrylic support matrix. Preferably the support is agarose. An ideal QAE agarose anion exchange resin is Q-SEPHAROSE® (Pharmacia, Piscataway, N.J.).

IL-10 produced by a mammalian cell culture system does not adsorb to a QAE anion exchange resin at the optimal pH of 8.0-8.3. Thus, IL-10 passes through a QAE column while most contaminating proteins are adsorbed if the mg protein/ml bed volume is below 4.

Acetylation of IL-10 contained within a fraction can be determined by first separating the variants of IL-10 by reverse-phase high performance liquid chromatography (HPLC). Mass spectrometry of the intact protein or trypsin-digested fragments can then be performed. Mass spectrum analysis would then indicate an increase in mass of the IL-10 or fragment thereof equivalent to the mass of an acetyl group if the IL-10 or fragment is acetylated. In addition, N-terminal sequence analysis of trypsin-digest fragments demonstrates a peak which matches an acetylated-lysine standard if the IL-10 is acetylated. The non-acetylated IL-10 produced in a prokaryotic system adsorbs weakly to the anion exchange resin as it passes through when the solution containing the protein is at a conductivity of 1.0-1.5 and pH 8.7. The acetylated dimers and contaminant host proteins adsorb more tightly to the column.

The IL-10-containing protein fractions obtained from the anion exchange column are subjected to hydroxyapatite chromatography in order to separate the different dimeric forms of IL-10 present in the fractions. This can be done by a fast flow method in which the anion exchange column is placed directly above the hydroxyapatite column so

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that the fractions from the anion exchange column are immediately loaded onto the hydroxyapatite column as they come off of the anion exchange column.

- 5           If the IL-10 is produced in a mammalian cell culture system, the hydroxyapatite column is equilibrated with a standard salt solution at a pH of about 8.1. A suitable buffer for this purpose is comprised of 20 mM Tris-Cl, and 20 mM NaCl, pH 8.1. The IL-10 containing fractions are loaded onto the hydroxyapatite column and eluted preferably with a  
10 20 bed volume linear gradient of a 150 mM potassium phosphate buffer at about a pH of 8.0. The elution is started with about a 6% concentration of the  $KPO_4$  buffer and increases gradually until a concentration of about 75% is reached.  $NaPO_4$  can also be used at the same concentration levels. The  $\Delta 0:\Delta 0$  IL-10 dimer elutes off at about a  
15 20-25% concentration of the 150 mM  $KPO_4$  buffer. The other two dimers elute off at about 30-35% of the 150 mM  $KPO_4$  buffer.  $\Delta 0:\Delta 2$  can then be separated from  $\Delta 2:\Delta 2$  preferably by doubling the length of the column and applying and reapplying the resultant fractions until the  $\Delta 0:\Delta 2$  and the  $\Delta 2:\Delta 2$  come out in separate fractions. Using hydroxyapatite  
20 chromatography, different IL-10 dimers present together in IL-10-containing fractions can be separated from each other. The fact that the different dimers have indeed been separated can be determined by N-terminal amino acid residue sequence analysis.
- 25           If the IL-10 is produced in a prokaryotic expression system, the truncated dimers are rare. However, non-covalently bonded IL-10 dimers must be separated from covalently bonded IL-10 dimers. This is done by hydroxyapatite chromatography. The hydroxyapatite column is equilibrated with a standard salt solution at a pH of about 7.4. A suitable  
30 buffer for this purpose is comprised of 20 mM Tris-Cl, and 20 mM NaCl, pH 7.4. The IL-10 containing fractions are loaded onto the hydroxyapatite column and eluted preferably with a 20 bed volume linear gradient of a 150 mM sodium phosphate buffer, pH 7.4. The elution is started with about a 5% concentration of the  $NaPO_4$  buffer and  
35 increases gradually until a concentration of about 100% is reached.  $KPO_4$  can also be used at the same concentration levels. The non-covalently bonded dimer elutes off first at 7-10 mS, and the covalently bonded dimer peaks at about 12 mS.

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The isolated IL-10-dimer-containing fractions obtained from the hydroxyapatite column are then subjected to gel filtration. Gel filtration is used to separate high and low molecular weight impurities including  
5 IL-10 monomer. Two particularly useful gels are SEPHACRYL S-200 HR<sup>®</sup> which has a fractionation range of 5 kDa to about 250 kDa for proteins, and SEPHACRYL S-100<sup>®</sup>, which has a fractionation range of 1 to 100 kDa for proteins. Other gels which have fractionation ranges from about 1 kDa to 600 kDa for proteins may also be used.

10

Variant forms of a protein differing in the N-terminal amino acid sequence can be separated using hydroxyapatite chromatography. A protein, monomeric or oligomeric, may be purified but still retain heterogeneity due to variants missing one or more N-terminal amino  
15 acids. These variants may be separated by hydroxyapatite chromatography. In order to effect these separations a number of experimental variables are examined. First is the phosphate concentration necessary to elute, and the gradient of the phosphate concentration. Secondary variables to be examined are the column  
20 length, protein loading, pH, net conductivity and low levels of divalent cations. Rechromatography under somewhat altered conditions is likely to improve the yield and purity of the variant forms.

The following examples are included to illustrate but not limit  
25 the present invention.

#### EXAMPLE 1

##### Purification of Human IL-10 from CHO - Cell Line Culture Medium

30

Chinese Hamster Ovary (CHO) cells were transfected with a vector containing the IL-10 gene and were grown in Iscove's Modified Dulbecco's Medium (IMDM) a basal medium containing salts, buffers, vitamins, amino acids, and glucose (Sigma, St. Louis, Missouri)  
35 supplemented with 5% NUSERUM V<sup>®</sup> a medium supplement containing 25% newborn calf serum, hormones growth factors and other nutrients (Collaborative Research) and HBCHO<sup>®</sup> a serum-free supplement containing bovine serum albumin, insulin, transferrin,

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fetuin, fatty acids, ethanolamine, and selenium (Irvine Scientific). The transfected CHO cells were grown in the cell medium at 37°C at a pH of 7.2. After five days of growth, a total of 177 liters of the cell culture supernatant liquid was drawn off, subjected to crossflow microfiltration and concentrated to about 17.6 liters by ultrafiltration. The CHO - cell culture supernatant was then diafiltered with 20 mM MES (2-[N-Morpholino]ethanesulfonic acid), 65 mM NaCl, pH 4. The resultant supernatant liquid was then subjected to the following chromatographic procedures, all of which were performed at 4°C.

10

#### Cation - Exchange Chromatography

15

The concentrated, diafiltered CHO-cell supernatant concentrate was loaded on a 5 x 28 cm S-SEPHAROSE<sup>®</sup> Fast Flow column equilibrated with 20mM MES, 70 mM NaCl pH 6.5. Approximately 100 mg protein/ml of bed volume was applied at a flow rate of 1.1 cm/min. The column was washed with 8.5 bed volumes of equilibration buffer. This was followed by elution with a 13 bed volume, 70-300 mM NaCl gradient at a reduced flow rate of 0.6 cm/min. The hIL-10 eluted at a distinct peak of A<sub>280</sub> at approximately 17 mS which corresponded to about 150 mM NaCl and it was the major protein eluted at 16-20mS. The fractions containing hIL-10 were concentrated and diafiltered (PELLICON<sup>®</sup>, 10 K membrane) with Buffer A, which was comprised of 20 mM Tris-Cl, 20 mM NaCl, pH 8.1.

Cation - exchange chromatography utilizing S-SEPHAROSE<sup>®</sup>, produced good adsorption and was therefore chosen as the first purification step. Using the conditions described above, 80-90% of the contaminant protein was not bound. Although hIL-10 was 1% of the initial protein, it was the major protein eluted at 16-20 mS and was purified 50-fold. See Table 1 below. Optimal conditions of pH, conductivity, flow rates, and column dimensions were determined by evaluating UV absorbance at 280 and 260 nm, protein concentration, ELISA values, SDS-PAGE, and Western blot results of numerous chromatographies.

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### Anion - Exchange Chromatography

The concentrated, diafiltered, IL-10-containing fractions obtained  
5 from the cation - exchange chromatography step were loaded on a 5 x 13  
cm Q-SEPHAROSE® Fast Flow column equilibrated with Buffer A. The  
protein loading was approximately 3.5 mg/ml bed volume at  
0.5 cm/min. The column was then washed with the Buffer A until the  
absorbance at A<sub>280</sub> was minimal. The protein which did not adsorb to  
10 the Q-SEPHAROSE® contained the hIL-10 and was pooled for direct  
loading onto hydroxyapatite.

Human IL-10 had little affinity for various anion exchange  
columns, showing minimal binding at pHs up to 8.1, and conductivities  
15 down to 4mS. This allowed Q-SEPHAROSE® chromatography in the  
flow-through mode where hIL-10 passed directly through the column,  
while most contaminating proteins were adsorbed if the mg protein/ml  
bed volume was kept below 4. Since there is no buffer adjustment of  
the Q-SEPHAROSE® pool prior to hydroxyapatite chromatography, the  
20 two columns can be connected in tandem so that the effluent of the Q-  
SEPHAROSE® column is loaded directly on the hydroxyapatite column.

### Hydroxyapatite Chromatography

25 The IL-10 containing fractions obtained from the  
Q-SEPHAROSE® column were loaded on a 2.6 x26 cm hydroxyapatite  
column and equilibrated with Buffer A in order to separate the IL-10  
dimers which were present in the fractions. The hydroxyapatite which  
was used was a ceramic hydroxyapatite from Pentax and distributed by  
30 American International Chemical Inc.. Ceramic hydroxyapatite is  
formed by heating, i.e., sintering, the hydroxyapatite crystals into beads.  
Standard, i.e., non-sintered, hydroxyapatite (Biorad) is also acceptable.  
The protein loading was at approximately 2.5 mg/ml bed volume at a  
flow rate of 0.6 cm/min. The column was washed with 5 bed volumes  
35 of a mixture of 94% Buffer A and 6% Buffer B. Buffer B was comprised  
of 150 mM KPO<sub>4</sub>, pH 8.0. The IL-10 was eluted with a linear gradient  
from 6% to 75% of Buffer B. The Δ0:Δ0 dimer eluted out at  
approximately 20-25% concentration of Buffer B. The Δ0:Δ2 and the

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$\Delta 2:\Delta 2$  dimers eluted out at approximately 30-35% concentration of Buffer B.

### Gel-Filtration Chromatography

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Separate concentrated hydroxyapatite pools containing the  $\Delta 0:\Delta 0$  IL-10 dimer (up to ~20 mg/ml) were loaded onto either a SEPHACRYL<sup>®</sup> S-200 HR or SEPHACRYL<sup>®</sup> S-100 HR column (2.6 x 85 cm) equilibrated and eluted with Buffer C which was comprised of 20 mM Tris-Cl, 150  
10 mM NaCl, pH 8.1. The sample loading volume was less than 4% of the bed volume and the flow rate was 0.1 cm/min. Peak fractions were pooled and stored at -20°C.

Gel filtration chromatography in either SEPHACRYL<sup>®</sup> S-200 HR  
15 or SEPHACRYL<sup>®</sup> S-100 HR revealed that hIL-10 displayed a molecular weight consistent with a dimeric form. The  $\Delta 0:\Delta 0$  dimer was the predominant form for all protein concentrations loaded (0.2-20 mg/ml of bed volume). Small amounts (<5%) of hIL-10 monomer were occasionally seen as a trailing shoulder on a A<sub>280</sub> profile, and these  
20 fractions were excluded from the pool.

The overall purification procedure yielded approximately 1.1 mg of at least 98% pure  $\Delta 0:\Delta 0$  human IL-10 per liter of cell culture medium. Purity was determined by sodium dodecyl sulfate-polyacrylamide gel  
25 electrophoresis (SDS-PAGE), Laemmli, U.K., *Nature*, 227 : 680 (1970). In addition, HPLC chromatography with either reversed-phase (C<sub>4</sub>) or with size exclusion ZORBEX<sup>®</sup> 250 showed only a single peak. The performance of each of the purification steps is shown in the following Table, in which results are an average from three purification runs of  
30 approximately 175 liters each of CHO cell supernatant (containing 5% Nu-Serum V).



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TABLE 1Purification of CHO hIL-10

Step	Absorbance (A <sub>280</sub> units)	Total		Yield <sup>a</sup> (%)	Purity <sup>b</sup> (%)
		Proteins (mg)	hIL-10 <sup>a</sup> (mg)		
cell culture	55,000	57,000	600	100	1.1
S-Sepharose	680	890	430	72	48
Q-Sepharose	190	360	290	48	81
Hydroxyapatite	130	200	210	35	92
Sephacryl S-200	73	180	200	33	98

5

<sup>a</sup> Concentration and yield of hIL-10 was based on an ELISA assay.

<sup>b</sup> Purity was determined as mg hIL-10(as determined by ELISA) / mg total protein (as determined by the Bradford Assay *supra*) x 100 for the cell culture concentrate, S-Sepharose pool, and Q-Sepharose pool. Purity was determined from SDS-PAGE by comparison of band intensity at varying protein amounts for the hydroxyapatite pool and the Sephacryl S-200 pool. In this technique known amount of the sample ranging from 0.005-25 µg were run in different lanes of an SDS-PAGE gel. The relative amount of contaminants seen at high loading were determine by comparison with the band intensity of IL-10 seen at low loadings.

10  
15EXAMPLE 2

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Purification of Human IL-10 from *E. coli*

*Escherichia coli* (*E. coli*) was transformed with an expression plasmid containing a gene encoding and expressing recombinant human Interleukin-10 (rhIL-10). The plasmid carried the strong hybrid *tac* promoter for transcription of the rhIL-10 [Zurawski, S.M. *et al.*, *J. Immunol.* 137:3354-3360 (1986)]. The *lpp* 3' coding and non-coding regions including the transcription terminator, lie downstream of the rhIL-10 coding region. Derived from pINIIIompA, this region of the *lpp* gene is believed to lend stability to mRNA upstream of it [Ghrayeb,

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J. *et al.*, *EMBO J.*, 3:2437-2442 (1984)]. The plasma carries a thermoinducible origin of replication, derived from pVU208, a copy control mutant derived from pCloDF13 [Hakkart, M.J.J. *et al.* 183:326-332 (1981)]. At elevated temperatures, *e.g.*, 42° C, plasmid copy number for a different plasmid carrying this origin of replication has been reported to increase from about 30 copies per chromosomal equivalent to several hundred [Andreoli, P.M. *et al.*, *J. Bacteriol.* 135:612-621 (1978)]. The plasmid carries the tetracycline resistance gene from pBR322 for plasmid maintenance [Sutcliffe, J.G., *C.S.H. Symp. Quant. Biol.* 43:77-90 (1978)].

5 The expression construct results in the rhIL-10 being produced intracellularly as insoluble inclusion bodies. The transformed *E. coli* were plated on agar containing 20 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl and 10 mg/L tetracycline. A single, well isolated colony was randomly picked from the agar plate and restreaked on a second agar

10 plate.

A master cell bank was then prepared by suspending two colonies from the freshly restreaked agar plate in 1 ml of LYM-1 medium containing 30 g/L casamino acids, 20 g/L yeast extract, 5 g/L KPO<sub>4</sub> [monobasic], 20 g/L glycerol, 1 g/L MgSO<sub>4</sub>, pH 7. This was then used to inoculate 100 ml of LYM-1 broth containing 10 mg/L tetracycline [LYM-1/Tc10] in a 300 ml baffled flask, which was then incubated at 30°C and shook using a rotary shaker at 300 rotations per minute (RPM) until the cell density reached Klett<sub>540</sub> of ~400 (early log phase). This culture was

20 then mixed 1:1 with 40% glycerol (v/v), yielding a final glycerol concentration of 20%. One ml aliquots were then dispensed into prelabeled cryovials quickly frozen under liquid nitrogen, and stored in a freezer set at -80°C prior to use.

30 A working cell bank was then prepared by thawing one vial of the master cell bank in air at room temperature which was then inoculated into 100 ml of LYM-1/Tc10 medium in a 300 ml baffled flask, which was then incubated at 30°C and shook using a rotary shaker at 300 RPM until the culture reached Klett of ~400 (early log). This culture was then

35 mixed 1:1 with 40% glycerol. One ml aliquots were then dispensed into prelabeled cryovials, quickly frozen under liquid nitrogen, and stored in a freezer set at -80°C.

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A 1.5 ml frozen vial of the working stock was thawed at room temperature. Approximately 0.5 ml of the working stock was transferred into a 2000 ml flask which contained 500 ml of LYM-1/Tc10 medium. The tetracycline was added just before inoculation. The flask  
5 was placed on a rotary shaker and shook at 300 RPM at 30°C. After 6.5-7 hrs a sample was removed from the flask for Klett<sub>540</sub> determination. The culture had a Klett <sub>540</sub> of 200-300. A 1000 liter fermentor containing 800 liters of LYM-3/Tc10 medium was then inoculated with the contents of the 2000 ml flask. LYM-3/Tc10 medium is comprised of 30 g/l Casein  
10 Digest-HyCase P (Sheffield), 20 g/l Yeast Extract-Type D (Bio Springer), 15 g/l Potassium Phosphate-Monobasic (Monsanto), 0.5 ml/l of a 30% suspension of SAG-471® (Union Carbide), 20 g/l Glycerin 99.7% (Univar), 1 g/l Magnesium Sulfate-7 H<sub>2</sub>O (PQ), 10 mg/l tetracycline (Sigma), 2 ml/l Iron Citrate Stock Solution comprised of 2 ml/l sulfuric  
15 acid, 15 g/l Sodium Citrate, 13.5 g/l Ferric Chloride Hexahydrate. The pH of the medium was adjusted to about 7 with a solution of 50% NaOH and a solution of 75% H<sub>3</sub>PO<sub>4</sub>. The temperature of the inoculated medium within the fermentor was maintained at 30°C ±0.5°C until 1000 ±100 Klett<sub>540</sub> was reached and then the temperature was elevated to  
20 38°C ±0.5°C for 14 hours. The dissolved oxygen concentration in the fermentor was maintained at a level greater than 40% of saturation by agitation.

The fermentation was harvested 14 hours after the temperature was  
25 elevated to 38°C by lowering the agitation, and chilling the medium to 5°C-15°C. The batch was centrifuged using a contained CSA-16 continuously desludging centrifuge at a feed flowrate of approximately 5-10 liters per minute (lpm). The flowrate was adjusted in order to obtain a clear centrate. One full desludge and one partial desludge (with  
30 bowl time set at 0.95 sec) was used to recover 800 liter of fermentation. A 40 ± 2kg cell pellet was recovered in the centrifugation step.

The 40 kg cell pellet recovered in the centrifugation step was homogenized using a Gaulin M12 homogenizer at an operating  
35 pressure of 7000-8000 psi for the equivalent of 6 passes. This was accomplished by recirculating the batch from a hold tank through the homogenizer and a glycol-cooled heat exchanger and back to the hold tank at a flowrate of 10 lpm for approximately 140 min. After the 140

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min homogenization, a sample of the homogenate was withdrawn and examined under a phase contrast microscope. This was done in order to assess cell breakage. If greater than 95% breakage, as estimated by microscopic evaluation, is not observed, then homogenization should  
5 be continued.

The homogenized cells were inactivated by mixing the homogenate with an equal volume of 4 M Guanidine Buffer comprised of 6.05 g/l TRIZMA-BASE® (Tris[hydroxymethyl]aminomethane) (Sigma), 1.90 g/l  
10 disodium EDTA dihydrate (Sigma), 58.4 g/l NaCl USP (Mallinckrodt), and 382 g/l Guanidine HCl (Sigma). The resuspension was held for 30 min. at 10-15°C under slow agitation. The inactivated resuspension was then centrifuged in a Sharples AS26SP centrifuge at a flowrate of 500 ml per minute and a centrifuge speed of 17000 rpm. A pellet containing  
15 inclusion bodies recovered in this step was frozen at -10°C. Inclusion bodies are aggregates that contain various *E. coli* host proteins, nucleic acids and other cellular debris, in addition to h-IL-10.

#### Unfolding IL-10

20 The inclusion body pellet which had been stored at -10°C was thawed for three days at 2-10°C in a cold room. The pellet was broken up and added into 20 liters of unfolding buffer. The unfolding buffer was comprised of 50 mM TRIZMA® (Tris[hydroxymethyl]aminomethane) (Sigma), 7 M guanidine HCl, and 4 mM dithiothreitol (DTT), pH 8.5.  
25 The inclusion body pellet was vigorously agitated with a polytron homogenizer to form a fine suspension. This suspension was then allowed to further solubilize by slow agitation for approximately 3 hours at 2-10°C.

#### Refolding IL-10

30 The soluble protein solution was then diluted approximately twenty five fold into a refolding buffer. The refolding buffer was comprised of 50 mM TRIZMA®, 0.12 M Guanidine HCl, 0.05 mM Glutathione (reduced), pH 8.5. The diluted refolding solution was immediately  
35 clarified by filtration; an oxidized glutathione solution was then added to a 0.45 mM final concentration and refolding continued for 10 -24 hours.

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### Concentration/Diafiltration

At the end of the refolding step, the solution was then clarified by filtration using a .45  $\mu$ m filter prior to ultrafiltration. Additionally, a  
5 filter may be placed in line to the ultrafilter to ensure clarity during ultrafiltration. The solution containing the refolded IL-10 was then concentrated approximately 10 fold. This was done with the ultrafiltration system Millipore PELLICON® ultrafilter with 10,000 nominal molecular weight PLGC membranes. The concentrate was  
10 then diafiltered to reduce the concentrate conductivity to approximately 6 mS. The diafiltration buffer was comprised of pH 8.5, 20 mM TRIS, 20 mM NaCl.

### Cation Exchange Chromatography

15 The concentrated solution containing the refolded h-IL-10 was adjusted to 20 mM BIS-TRIS, pH 6.5 by the addition of 1 M BIS-TRIS and 4N HCl. The solution was then clarified by filtration. The clarified feed solution containing approximately 1.2 mg of protein/ml was then  
20 applied to a 12 liter (12 cm x 36 cm diameter) S-SEPHAROSE® Fast Flow sulfonate column (Pharmacia, Piscataway, New Jersey) at a rate of 1 cm/min. The column had been pre-equilibrated with ten bed volumes of a pH 6.5, 20 mM BIS-TRIS, 0.065 M NaCl buffer, pH 6.5 at a rate of 1 cm/min. Elution was performed with a 20 column volume gradient in  
25 the range of 0.065-0.4 M NaCl, 20 mM BIS-TRIS, pH 6.5 buffer at a rate of 0.5 cm/min. The h-IL-10 peak fractions of the elution profile were determined by A<sub>280</sub> and verified by typical pH and conductivity ranges. The fractions containing the h-IL-10 eluted at 11-18 mS, 100-170 mM NaCl and were pooled together for further processing.

30

### Anion Exchange Chromatography

The pooled fractions from the cation exchange chromatography process containing the h-IL-10 were concentrated to 0.5 column  
35 volumes with an ultrafiltration system Millipore PELLICON® ultrafilter with 10,000 n.mol.wt. PLGC membranes. The concentrate was then diafiltered to approximately 1.5 mS using a 10 mM TRIS buffer pH 8.7. The pH of the diafiltered concentrate was adjusted to pH 8.7 with

- 20 -

HCl or NaOH. The solution containing approximately 13 mg/ml of protein was applied to a 6 liter (18 cm diameter x 23.5 cm) quaternary ammonium column Q-SEPHAROSE® Fast Flow (Pharmacia) at a flow rate of 0.5 cm/min. pre-equilibrated with 10 mM TRIS, 8 mM NaCl, pH 8.7 buffer. The h-IL-10 has a differential attraction to the resin versus the impurities contained within the fractions and was separated on the isocratic elution and collected in the column effluent with 10 mM TRIS, 8 mM NaCl, pH 8.7 buffer. The acetylated homodimers and acetylated heterodimers adsorbed more strongly to the resin, and thus were separated from the non-acetylated homodimers. The non-acetylated h-IL-10 peak fractions of the elution profile as determined by A<sub>280</sub> were pooled for further processing.

#### Hydroxyapatite Chromatography

The pool containing the h-IL-10 obtained from the anion exchange chromatography step was applied to a 4 liter (26 cm x 14 cm diameter) hydroxyapatite column (e.g., Ceramic Hydroxyapatite, Biorad MACRO-PREP) pre-equilibrated with 20 mM TRIS, 20 mM NaCl, pH 7.4 buffer. The column wash was performed by decreasing the amount of 20 mM TRIS buffer from 100% to 95% and increasing the level of pH 7.4, 0.15 M sodium phosphate buffer 0% to 5% for approximately 4 column volumes. The elution was performed by increasing the percent of phosphate buffer during a 17 bed volume elution gradient from 5% to 100%. The non-covalently bonded dimer eluted at 7-10 mS, while the covalently bonded dimer peaked at 12 mS. The h-IL-10 peak fractions as determined by A<sub>280</sub> were pooled for further processing.

#### Gel Filtration Chromatography

The pool from the hydroxyapatite process step containing the non-acetylated, non-covalently bonded IL-10 dimer was concentrated on an ultrafiltration system containing a 10,000 n. molecular weight membrane. The concentrate was applied to a gel filtration column which was a 14.8 liter (96 cm x 14 cm diameter) SEPHACRYL® S-200 HR, pre-equilibrated with 10 mM TRIS buffer, pH 7.4. The column was eluted with a pH 7.4, 10 mM TRIS buffer. The h-IL-10 peak fractions of the elution profile as determined by A<sub>280</sub> were pooled for further

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processing. The gel filtration pool was filtered through a 0.2  $\mu$ m filter. The filtrate, the final purified bulk drug, was stored at -20°C.

Gel filtration chromatography in either SEPHACRYL® S-200 HR or SEPHACRYL® S-100 HR revealed that hIL-10 displayed a molecular weight consistent with a dimeric form. The non-acetylated, non-covalently bonded dimer was the predominant form for all protein concentrations loaded (0.2-20 mg/ml of bed volume). Small amounts (<5%) of hIL-10 monomer were occasionally seen as a trailing shoulder on the A<sub>280</sub> profile, and these fractions were excluded from the pool.

The performance of each of the purification steps is shown in the following Table 2.

TABLE 2

Purification of hIL-10 Expressed by *E. coli*

Step	Total				
	Absorbance (A <sub>280</sub> units)	Proteins (mg)	hIL-10 <sup>a</sup> (mg)	Yield <sup>a</sup> (%)	Purity <sup>a</sup> (%)
Refolding <sup>b</sup>	183,000	161,000	44,800	100	45
S-Sepharose	21,700	42,000	28,900	65	81
Q-Sepharose	6,860	18,900	17,300	39	99
Hydroxyapatite	5,670	16,500	13,700	31	99
Sephacryl S-200	4,950	14,400	12,000	27	99

<sup>a</sup> Concentration, yield and purity of hIL-10 was based on Reverse Phase HPLC analysis.

<sup>b</sup> After concentration, diafiltration, and pH adjustment.

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EXAMPLE 3Hydrophobic Interaction Chromatography of Refolded Human IL-10

- As an alternative to diafiltration of the refolded IL-10 the following procedure can be used to remove the refolding and denaturing buffers. Inclusion bodies containing human IL-10 which had been expressed in *E. coli* were resuspended in a 10:1 (v/w) ratio of unfolding buffer to inclusion bodies. The unfolding buffer was comprised of 6M Guanidine Hydrochloride (GdnHCl), 4 mM dithiothreitol (DTT), 50 mM Tris pH 8.5, 1 mM ethylene diamine tetraacetic acid (EDTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF). The buffer containing the inclusion bodies was incubated with stirring for three hours at 4° C producing unfolded, denatured human IL-10.
- The unfolded denatured IL-10 was diluted 100 fold into a refolding buffer containing 50 mM Tris pH 8.5, 1 mM EDTA, 0.5 M final concentration of GdnHCl, 4.17 mM reduced glutathione, 0.83 mM oxidized glutathione, 2 mM benzamidine and incubated for 17 hours at 4° C.
- After refolding, the mixture was filtered through a 0.45 µm filter, brought to 25% ammonium sulfate concentration and filtered again. The filtrate was applied to a Butyl-Toyopearl 650 M Hydrophobic Interaction (TosoHaas) column (pre-equilibrated into 25% ammonium sulfate, 20 mM Tris pH 8.5), at a ratio of 0.25 to 0.5 grams of inclusion bodies per ml of resin at a linear flow rate of 1 cm/min. In this step, the IL-10 was bound to the column while many of the protein and most of the non-protein contaminants such as the refolding process reagents such as glutathione and GdnHCl, low molecular weight contaminants, fragments of *E. coli* cell components etc. which are characteristic of refolding mixtures passed through the column. The bound human IL-10 was then eluted isocratically with 20 mM Tris pH 8.5, 20-50 mM NaCl. Twenty one-bed volume fractions were collected. The human IL-10 began to elute two bed volumes into the isocratic gradient. Generally, fractions 2-15 were pooled. The hydrophobic interaction pool can then be further processed for further purification.



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While the present invention has been described in conjunction with the specific embodiments set forth above, many alternatives, modifications and variations thereof will be apparent to those of ordinary skill in the art. All such alternatives, modifications and variations are intended to fall within the spirit and scope of the present invention, which is only to be limited by the claims.

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## WHAT IS CLAIMED IS:

1. A method for purifying human Interleukin-10 (IL-10) contained within a solution comprising:  
5  
    (a) subjecting the solution containing IL-10 to cation exchange chromatography thereby obtaining fractions containing IL-10;  
  
    (b) subjecting the IL-10-containing fractions from step (a) to anion  
10 exchange chromatography thereby obtaining fractions containing IL-10;  
  
    (c) subjecting the IL-10-containing fractions from step (b) to hydroxyapatite chromatography thereby obtaining fractions containing a single isolated dimer of IL-10.  
15
2. The method of claim 1 wherein the cation exchange chromatography column from step (a) is comprised of sulfonate exchange groups attached to a support matrix.
- 20 3. The method of claim 2 wherein the support matrix is agarose.
4. The method of claim 1 wherein the anion exchange chromatography column is comprised of quaternary amino ethyl exchange groups attached to a support matrix.  
25
5. The method of claim 4 wherein the support matrix is agarose.
6. The method of claim 1 further comprising applying the IL-10-containing fractions obtained from step (c) of claim 1 to a gel filtration  
30 chromatography column to obtain dimeric IL-10 substantially free of high and low molecular weight impurities.
7. The method of claim 6 wherein the gel has a fractionation range of from 1 to 600 kDa.  
35
8. The method of claim 1 wherein the IL-10 was produced by a prokaryotic expression system, denatured from inclusion bodies and refolded prior to purification.

- 25 -

9. The method of claim 1 wherein IL-10 is secreted in a cell culture  
5 medium from eukaryotic cells.
10. A method for separating different dimers of IL-10 present in an  
IL-10 containing protein fraction comprising:  
subjecting the IL-10 containing fraction to hydroxyapatite  
10 chromatography under conditions wherein the different IL-10 dimers  
are separated from each other.
11. The method of claim 10 wherein the IL-10 dimers present in the  
protein fraction are  $\Delta 0:\Delta 0$ ,  $\Delta 0:\Delta 2$  and  $\Delta 2:\Delta 2$  IL-10 dimers and the  $\Delta 0:\Delta 0$   
15 dimers are collected.
12. The method of claim 10 wherein the IL-10 dimers present are non-  
covalently bonded IL-10 dimers and covalently bonded IL-10 dimers,  
wherein the non-covalently bonded IL-10 dimers are collected.  
20
13. A method for separating different dimers of a protein contained  
within a protein fraction wherein the different dimers have different N-  
terminal amino acid sequences comprising:  
subjecting the protein fraction to hydroxyapatite chromatography  
25 under conditions wherein the different dimers of the protein are  
separated from each other.
14. A method for separating variants of a protein contained within a  
protein fraction wherein the variants of the protein have different N-  
30 terminal amino acid sequences comprising:  
subjecting the protein fraction to hydroxyapatite chromatography  
under conditions wherein the variants of the protein are separated from  
each other.  
35
15. A method for separating non-acetylated homodimers of IL-10 from  
acetylated IL-10 homodimers and from acetylated IL-10 heterodimers  
contained within a solution comprising:

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applying the solution to anion exchange chromatography under conditions in which the non-acetylated homodimers are separated from the acetylated homodimers and from the acetylated heterodimers.

# INTERNATIONAL SEARCH REPORT

Int. Application No  
PCT/US 94/01909

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 5 C07K3/28 C07K3/18

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 5 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J.EXP.MED. vol. 170 , December 1989 pages 2081 - 2095 FIORENTINO, D.F. ET AL. 'Two types of mouse T helper cell' cited in the application * page 2089, "Partial purification..."; fig. 7 *	1-15
Y	WO,A,91 00349 (SCHERING CORPORATION) 10 January 1991 cited in the application * p. 27, lines 12-23; claim 14 *	1-15
Y	EP,A,0 061 139 (MAX-PLANCK-GESELLSCHAFT) 29 September 1982 * p. 40-49 *	1-15

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
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- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 94/01909

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>LYMPHOKINE AND CYTOKINE RESEARCH vol. 11, no. 2 , 1992 pages 87 - 93 HISATSUNE, T. ET AL. 'A suppressive lymphokine derived from Ts clone 13G2 is IL-10' * p. 88-89: "Purification of TGIF ..."; table 1 *</p> <p>-----</p>	1-15

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 94/01909

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		CA-A- 2062763	29-12-90
		CN-A- 1051393	15-05-91
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		EP-A- 0567450	03-11-93
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		CA-A- 1188242	04-06-85
		JP-A- 58049319	23-03-83
		US-A- 4512971	23-04-85
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